Journal of Virological Methods 204 (2014) 73–80

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Analytical performance of newly developed multiplex human papillomavirus genotyping assay using Luminex xMAP™ technology (Mebgen™ HPV Kit)

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Article history:
Received 23 September 2013
Received in revised form 11 April 2014
Accepted 15 April 2014
Available online 24 April 2014

Keywords:
HPV
HPV genotyping
Multiplex PCR
Luminex
Cervical cancer

A B S T R A C T

Regional differences in human papillomavirus (HPV) genotypes and the presence of mixed HPV infections may affect adversely the efficacy of the HPV vaccine. Therefore, a simple and high-throughput HPV genotyping system is required. Recently, a novel HPV genotyping kit (the Mebgen™ HPV kit) was developed. This kit uses multiplex PCR and Luminex xMAP™ technology to detect 13 types of high-risk HPVs and an internal control in a 96-well format. In the present study, the analytical performance of the kit was examined using HPV plasmid DNA. All 13 types of HPVs were detected with a minimum detection sensitivity of 250 copies/test, and highly specific signals were observed. HPV 16 plasmid was detected in samples containing mixtures with other HPV-type plasmids in ratios ranging from 1:1 to 1:1000. No cross reactivity was observed with DNA from 27 types of other infectious microbes. A clinical evaluation was carried out using cervical samples from 356 patients with persistent abnormal smears diagnosed at mass public health screenings for cervical cancer. The samples were preserved in Tacas™ medium until analysis. HPV was detected in 162 (45.5%) samples including 110 (67.9%) with single infections and 52 (32.1%) with multiple infections. The type distribution of the 13 high-risk HPVs was as follows: 28.4% HPV 16, 11.7% HPV 18, 6.8% HPV 31, 3.1% HPV 33, 3.7% HPV 35, 9.3% HPV 39, 1.9% HPV 45, 8.6% HPV 51, 37.0% HPV 52, 9.3% HPV 56, 16.7% HPV 58, 3.7% HPV 59, and 1.9% HPV 68. To evaluate sample stability over time, changes in the detection of HPV DNA derived from HeLa and SiHa cells were measured in 3 types of liquid-based cytology media. HPV DNA was detected in Tacas and Thinprep™ samples after storage at 4 °C or 30 °C for 4 weeks and within 1 week of collection in Surepath™ samples. These results suggest that this newly developed HPV genotyping kit is suitable for use in both clinical applications and large-scale epidemiological studies.

1. Introduction

In many countries, the Papanicolaou-stained (Pap) cytological smear has been used conventionally for detecting cervical cancer precursor lesions, and its use has reduced successfully the incidence of cervical cancer. However, a limitation of this test is its relatively low sensitivity of 50% (Baldwin et al., 2003; Michalas, 2000; Wright, 2007). The development of cervical cancer is known to be associated with human papillomavirus (HPV) infection (Burd, 2003; zur Hausen, 2002). More than 100 types of HPV have been identified from differences in the DNA sequence of the L1 open reading frame,

Abbreviations: HPV, human papillomavirus; PCR, polymerase chain reaction; DNA, deoxyrbonucleic acid.

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http://dx.doi.org/10.1016/j.jviromet.2014.04.010

and approximately 40 types of HPV have been reported to infect the genital tract (Bosch et al., 2002; Munoz et al., 2003). Twelve high-risk HPV types have a causal link with cervical cancer (Bouvard et al., 2009), in particular, HPV 16 and HPV 18 are associated strongly with a higher risk for the progression of cervical cancer (Kjaer et al., 2010).

Recently, HPV DNA tests have been developed for early cancer screening using molecular biology techniques, and these can identify women at increased risk of developing cervical cancer. In 2009, the American society for colposcopy and cervical pathology implemented clinical guidelines for individualized examinations that focused on the combination of an HPV DNA test and a Pap test in cervical cancer screening. In 2012, these guidelines were updated to improve the management of abnormal screening tests, including discordant HPV and Pap co-testing results, by defining when routine screening is required after initial assessment and expanding the management guidelines for adolescents to include women up to the age of 25 years. In 2010, the Japanese government approved the use of the HPV DNA test as a triage test for patients with "atypical squamous cells of undetermined significance", and in 2012, HPV genotyping was approved for women diagnosed with cervical intraepithelial neoplasia grade 1 or 2 on colposcopic biopsy.

In a recent study of a Columbian population screened using an HPV genotyping test, the multiple infection rate was found to be higher and the disease more advanced (Ili et al., 2011). HPV genotyping appears to be suitable highly for large-scale epidemiological and vaccination studies as well as for diagnostic screening and determining clinical treatment strategies (Castle et al., 2009; Chen et al., 2011). Furthermore, HPV genotyping is valuable for investigating the epidemiology of HPV and for monitoring the efficiency of HPV vaccination trials (Schmitt et al., 2006).

The current US food and drug administration-approved Hybrid capture™ 2 (HC2) test (Qiagen, Gaithersburg, MD, USA) lacks the ability to specifically genotype HPV and only determines samples as positive for high-risk HPV types. The Mebgen™ HPV kit (MBL, Nagoya, Japan) has been listed in the national health insurance report as a newly commercialized HPV genotyping method. This kit is able to detect 13 types of high-risk HPVs (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68). It uses the PCR-reverse sequence-specific oligonucleotide probe (PCR-rSSO) method with Luminex xMAP™ (Luminex, Austin, TX, USA) technology in a 96-well format and incorporates human β-globin genes as an internal control. With this method, the HPV DNA and β-globin genes in specimens are amplified by type-specific primers used for multiplex PCR. Subsequently, PCR products are detected by synthetic oligonucleotide probes with complementary sequences. Luminex is a high-throughput assay system that uses flow cytometry and bead sets coupled to probes that recognize and bind PCR products. Each bead set is characterized by a discrete spectral reference given by the combination of red and infrared fluorophores within the spheres. The target DNA is tagged fluorescently with streptavidin labeled with R-phycoerythrin (SAPE), and the beads are analyzed individually with a red laser that recognizes the bead set and a green laser that provides a quantitative readout of the bound target (Dunbar et al., 2003). To evaluate the analytical performance of this newly developed HPV genotyping kit, HPV plasmid DNA and clinical samples were examined under several conditions.

2. Materials and methods

2.1. Control plasmid DNA

Recombinant plasmid DNA was prepared for use as a positive control to determine the analytical sensitivity and specificity of the HPV genotyping kit. The plasmid DNA was prepared by inserting the synthesized L1 and L2 regions of each HPV genotype into pIDTBluev2 (IDT) vectors, and the concentration was adjusted to 50 copies/μl with TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) and mixed with 2 ng/μl human genomic DNA. Human genomic DNA at a concentration of 2 ng/μl in TE buffer was used as an HPV negative control. The L1 and L2 regions of the 13 high-risk HPV types were synthesized on the basis of the DNA sequences obtained from Genbank [accession nos.: HPV 16 (K02718), HPV 18 (X05015), HPV31 (J04353), HPV 33 (M12732), HPV 35 (M74117), HPV39 (M62849), HPV 45 (X74479), HPV 51 (M62877), HPV 52 (X74481), HPV 56 (X74483), HPV 58 (D90400), HPV 59 (X77858), and HPV 68 (M73258)]. All plasmid DNA constructs were confirmed by PCR-amplified DNA sequencing.

2.2. Clinical samples

All 356 samples were obtained from patients (mean age, 40.4 years; median, 38.5 years; range, 20–85 years) with persistent abnormal smears diagnosed during public health screening for cervical cancer at Kanazawa University Hospital (Ishikawa, Japan) and Toho University Hospital (Tokyo, Japan). Samples were collected using the Cervex-brush™ combi (Rovers Medical Devices, Oss, The Netherlands) and were stored in Tacas™ gyn preservative solution (MBL, Tokyo, Japan) at 4 °C for 1 month. The collection of all cervical samples was approved by and conducted in accordance with the human research ethics committee of each hospital.

2.3. Mebgen HPV assay

2.3.1. DNA extraction

DNA was extracted from 1.0 ml of each sample using the Smi test EX-R&D DNA extraction kit (MBL) according to the manufacturer's instructions. Template DNA was stored at −20 °C until use.

2.3.2. Multiplex PCR

Extracted DNA was amplified using multiplex PCR with a set of 14 primers recognizing HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68 and β-globin (Table 1). The amplification was performed in 96-well plates (Thermo fast 96 PCR plate; Thermo Fisher Scientific, Yokohama, Japan) in a final volume of 25 μl [20 μl master mix, including primers, Taq DNA polymerase, and Uracil-DNA-glycosylase (UDG), and 5 μl extracted DNA]. Reactions were heated for 5 min at 40 °C and 2 min at 95 °C, followed by 35 repeating cycles of 94 °C for 30 s, 61 °C for 60 s, 72 °C for 30 s, and 72 °C for 7 min before a final extension step at 94 °C for 10 min. PCR was performed in the Gold 96-well gene amp PCR system 9700 (Applied Biosystems, Foster City, CA, USA).

2.3.3. Hybridization and fluorescent labeling

Following the PCR amplification, 5 μl of PCR products and 25 μl of hybridization solution containing 15 types of probe (Table 1)-coupled beads of each set were transferred to 96-well plates (Thermo fast 96 plate non-skirted low profile; Thermo Fisher Scientific). Hybridization was performed at 95 °C for 2 min followed by 50 °C for 25 min using the Gold 96-well gene amp PCR system 9700. After hybridization, wash buffer was added to each well, and the plates were centrifuged at 2000 *g* for 1 min. Subsequently, supernatants were removed. After further washing steps, the beads were resuspended in 50 μl of R-phycoerythrin solution at 50 °C for 15 min.

2.3.4. Fluorescence measurement

The beads were analyzed for internal bead color and R-phycoerythrin reporter fluorescence on the Luminex 100/200. The median reporter fluorescence intensity (MFI) of at least 50 beads was computed for each bead set in the sample. The cut-off value for

Table 1
Oligonucleotide primers and probes for multiplex PCR using the Mebgen HPV kit.

HPV type	Region	Primer sequence (5′ → 3′)		Amplimer length (bp)	Probe sequence (5′ → 3′)
HPV 16	L1	Forward Reverse	GATACTACACGCAGTACAAATATGTC CCATGTCGTAGGTACTCCTTAAAG	98	TTCTGAAGTAGATATGGCAGCAC
HPV 18	L1	Forward Reverse	CGCAGTACCAATTTAACAATATGTGC CAAATCATATTCCTCAACATGTCTGC	108	TTTGGTAGCATCATATTGCCCAGG
HPV 31	L1	Forward Reverse	TACCACACGTAGTACCAATATGTCTG ATTCCTCACCATGTCTTAAATACTCTT	104	TGCTGCAATTGCAAACAGTGATAC
HPV 33	L1	Forward Reverse	GGATACCTATAGGTTTGTTACCTCT GTATATTTACCTAAGGGGTCTTCCT	90	TTTGACACGTAATAGCCTGAGAG
HPV 35	L1	Forward Reverse	CACATATCGCTATGTAACATCACAGG TAATGGATCATCTTTAGGTTTTGGTG	76	ACTTGTCAAAAACCCAGTGCAC
HPV 39	L1	Forward Reverse	CTGCCAGTTTGGTAGACACTTAC TCCAAACTAAACTTTTCCCTTAAGTC	142	AACTTTAGACCGTCATATGGAT TATGACGGTCTAAAGTTTTGGAA[a]
HPV 45	L1	Forward Reverse	CGTTTTGTGCAATCAGTTGCTG GGAGGAAAATTTTTCCTTTAGGTCA	117	ATATGGATCCTGCTTTTCTGG
HPV 51	L1	Forward Reverse	CCTGTGTTGATACTACCAGAAGTAC GCTTAAAGTTACTTGGAGTAAATGT	90	AGCAGTGGCAGTGCTAATAGT
HPV 52	L2	Forward Reverse	AGGTTTGGGTATAGGTACAGGTG TGGACGTGGTAATACTACTAGTGG	95	GCACATAGCCTGCCCTACCA
HPV 56	L1	Forward Reverse	GGGGTAATCAATTATTTGTTACTGTAG CTAAGGTACTGATTAATTTTTCGTGC	118	GTACTGCTACAGAACAGTTAAGTA
HPV 58	L1	Forward Reverse	GGACACATATAGATTTGTTACCTCC TTCCTTTAAGTTAACCTCCCAAAAAG	115	GGTGCTGTTTTTTGGCAAGTA
HPV 59	L1	Forward Reverse	GCTAGTTTAGTTGACACATACCGT AACTTTAGTTTGTCATAAGGGTCCT	104	CTGTTTAACTGGCGGTGCGG
HPV 68	L1	Forward Reverse	TGTTGTGGATACCACTCGCAGT TCCTCAACATGCCTAATATATTCC	114	CAGCTGATTCAGTAGTAGTAGAC
β-Globin		Forward Reverse	CAACTTCATCCACGTTCACC GAAGAGCCAAGGACAGGTAC	268	CTAGGGTGTGGCTCCACAGG

[a] Two types of probes were conferred in HPV 39.

a positive result was set at three times the MFI value of the negative control. HPV genotypes were generated automatically using the xPonent™ software (Luminex) that recognizes fluorescent peaks according to specific color and size.

2.4. Cloning and sequencing

To evaluate the genotypic specificity of the kit, 52 samples comprising 4 samples of each of the 13 types of HPV were selected randomly from 132 HPV-positive samples among the 356 clinical samples. This approach eliminated concerns about multiple HPV infection in this cloning and sequencing procedure. One HPV type-specific primer was used per sample of DNA. The PCR products were extracted using Suprec™-EZ (Takara Bio, Otsu, Japan) and cloned directly into pCR2.1 Topo TA cloning™ vector (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The cloned plasmids were sequenced with an ABI Prism™ 3100 genetic analyzer (Applied Biosystems). HPV-DNA types were confirmed using the basic local alignment search tool (BLAST) database on the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.5. Sample stability study

To examine the influence of sample storage conditions on HPV genotyping, changes in MFI over time were measured in 3 types of liquid-based cytology media. HeLa cells (containing HPV 18) and SiHa cells (containing HPV 16) were obtained from the American Type Culture Collection (Manassas, VA, USA) and were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Each cell type was maintained in a vial of liquid-based cytology media at a concentration of 1×10^5 cells/ml. To determine the optimal preservation period, DNA was extracted after preservation for 1–4 weeks at 4 °C or 30 °C. The liquid-based cytology media used were as follows: the Tacas medium as an example of an ethanol-based fixative, the Thinprep™ medium (Hologic, Bedford, MA, USA) as an example of a methanol-based fixative, and the Surepath™ medium (BD, Franklin Lakes, NJ, USA) as an example of an ethanol-based fixative including formaldehyde.

3. Results

3.1. Analytical performance

The specificity of the bead-coupled oligonucleotide probes was determined by hybridizing control plasmid DNA obtained from all 13 HPV genotypes and β-globin to a bead mixture comprising all specific probes. All samples were tested in 3 separate assays. Highly specific signals were observed for all 13 HPV type-specific probes with the respective control plasmid DNA (Table 2).

The cut-off value of MFI was calculated from the measurement data of 756 samples (three lots of 13 control plasmid DNA samples and human genomic DNA solutions were measured 18 times). The largest sample value of 10 standard deviations above the average negative sample value was 1382 for the HPV 39 bead, followed by 682 for the HPV 58 bead. The largest maximum sample value was 363 for the HPV 59 bead, followed by 758 for the HPV 39 bead. The smallest minimum sample value of 3 standard deviations below the average positive sample value was 1600 for the HPV 68 bead, followed by 5041 for the HPV 39 bead. The smallest minimum sample value was 2792 for the HPV 68 bead, followed by 5574 for the HPV

Table 2
Specificity and reproducibility of multiplex PCR products using control plasmids.

Plasmid	Beads	Median fluorescence intensity (MFI)															
		HPV type															β-Globlin
		16	18	31	33	35	39	39	39[b]	45	51	52	56	58	59	68	
HPV 16	n1	**7344**[a]	50	79	28	26	99	29	128	34	69	36	51	44	41	37	56
	n2	**7731**	43	25	52	29	143	27	169	62	77	40	61	69	56	33	87
	n3	**8354**	62	40	38	34	127	46	173	52	69	42	47	84	66	41	79
HPV 18	n1	29	**11,685**	39	36	65	113	41	154	58	87	44	51	107	62	46	97
	n2	52	**13,202**	64	52	58	148	35	183	59	98	65	70	88	71	40	106
	n3	20	**10,953**	51	32	49	113	19	132	52	77	43	51	70	53	34	69
HPV 31	n1	37	55	**10,814**	45	46	107	36	143	55	86	49	51	65	62	33	69
	n2	29	51	**11,271**	48	43	129	41	170	66	72	50	42	87	86	30	90
	n3	26	67	**10,889**	53	56	151	21	172	58	72	41	53	88	73	31	94
HPV 33	n1	48	39	50	**9353**	49	132	29	161	60	68	56	41	108	83	26	86
	n2	37	32	39	**8961**	51	128	38	166	48	66	31	47	95	54	26	72
	n3	35	70	56	**9003**	48	117	21	138	43	58	38	50	120	64	25	94
HPV 35	n1	53	40	69	56	**6394**	140	38	178	52	78	57	23	85	72	38	86
	n2	36	60	55	66	**6053**	136	42	178	63	87	59	63	69	74	49	105
	n3	28	47	43	54	**6101**	134	49	183	59	77	47	48	61	67	26	90
HPV 39	n1	38	57	52	54	37	**5259**	**2448**	**7706**	87	72	49	48	59	57	36	75
	n2	60	60	42	35	32	**5269**	**2281**	**7550**	70	60	44	53	84	47	28	104
	n3	55	70	43	45	55	**5045**	**2467**	**7511**	59	80	53	53	69	72	15	78
HPV 45	n1	53	50	64	60	42	145	45	190	**8185**	66	57	45	90	64	29	107
	n2	26	58	46	47	34	130	41	171	**7763**	61	57	40	64	55	33	92
	n3	51	47	55	53	46	157	31	188	**7519**	50	44	51	87	75	30	87
HPV 51	n1	31	77	65	57	30	132	44	176	42	**8552**	57	58	60	93	24	88
	n2	53	57	66	66	40	152	35	187	66	**9101**	49	60	69	79	39	95
	n3	27	59	50	60	44	145	46	191	76	**9504**	41	59	91	80	54	98
HPV 52	n1	42	59	58	45	41	165	33	198	66	75	**5914**	47	75	73	19	85
	n2	34	51	57	60	42	140	39	179	74	72	**6165**	55	65	70	33	92
	n3	58	74	47	55	41	148	47	195	67	86	**6031**	57	79	72	45	123
HPV 56	n1	52	60	64	57	29	192	39	231	68	72	50	**9005**	64	49	49	83
	n2	41	57	68	53	35	142	34	176	87	81	57	**8627**	75	72	39	85
	n3	45	50	59	35	44	124	44	168	34	80	29	**7830**	62	61	27	83
HPV 58	n1	40	50	60	78	41	142	32	174	81	91	50	60	**9647**	76	15	80
	n2	26	44	46	62	29	133	26	159	96	71	58	54	**10,050**	56	22	96
	n3	27	57	51	65	21	133	18	151	104	95	33	52	**10,080**	70	38	97
HPV 59	n1	41	35	52	44	31	119	31	150	61	69	54	44	77	**9644**	30	82
	n2	50	56	63	47	50	149	27	176	44	88	34	55	76	**10,072**	31	72
	n3	49	56	63	55	52	147	33	180	68	88	60	45	104	**10,364**	39	60
HPV 68	n1	46	44	54	42	42	144	42	186	74	81	56	67	83	87	**7951**	103
	n2	24	56	58	43	52	160	27	187	58	86	41	59	79	60	**7457**	100
	n3	38	47	63	32	43	156	49	205	66	59	62	82	70	56	**7635**	101
β-Globlin	n1	55	77	79	78	74	196	65	261	84	96	76	68	100	103	40	**3855**
	n2	39	57	62	68	55	157	49	206	64	61	57	66	87	78	47	**2798**
	n3	40	65	56	53	57	166	40	206	57	69	59	58	80	76	42	**2767**

[a] Specific signal (bold) is given as MIF.
[b] Total value of MFI with two types of HPV 39-specific probes.

Table 3
Detection limit analysis for each HPV type.

	Cut-off value		Copy number of plasmids per test				
			62.5 copies	125 copies	250 copies	500 copies	1000 copies
HPV 16	1000	MFI	2245.3	3183.4	4677.3	4882.5	7687.5
		CV%	22.1%	29.3%	10.0%	14.4%	6.0%
HPV 18	1000	MFI	4781.9	8815.3	10,084.5	10,776.3	10,333.3
		CV%	46.2%	4.1%	5.9%	6.3%	10.3%
HPV 31	1000	MFI	2830.3	3410.3	4859.3	5382.4	8191
		CV%	10.1%	18.7%	12.0%	10.2%	8.4%
HPV 33	1000	MFI	4941.8	6957.8	7736.7	8757.5	9939.5
		CV%	21.5%	16.7%	3.7%	5.4%	3.5%
HPV 35	1000	MFI	6828.4	8324.7	9806.3	9895.8	10,861.2
		CV%	17.4%	14.8%	2.8%	6.6%	5.7%
HPV 39	2000	MFI	4380.3	4414.8	5953	6213.3	7140.6
		CV%	12.6%	14.5%	6.4%	8.3%	10.3%
HPV 45	1000	MFI	4338.8	6245.7	6945.8	7884.3	9036.2
		CV%	13.8%	6.8%	5.9%	4.0%	2.6%
HPV 51	1000	MFI	2215.3	2752.3	4123	5609.3	8500.3
		CV%	36.3%	33.0%	10.0%	8.6%	14.5%
HPV 52	1000	MFI	6255.3	8131.3	10,753.9	11,299.7	15,786.3
		CV%	26.9%	16.6%	10.9%	5.9%	3.5%
HPV 56	1000	MFI	4535.8	4731.9	6694.2	7105	9148.1
		CV%	14.7%	10.6%	3.5%	5.3%	2.1%
HPV 58	1000	MFI	2348.3	2282	4655.7	4314.5	8778.8
		CV%	20.0%	11.2%	16.3%	8.9%	2.9%
HPV 59	1000	MFI	7073.9	6941.4	7930.1	8668.4	10,321.9
		CV%	11.8%	6.9%	8.3%	3.7%	2.0%
HPV 68	1000	MFI	693	1604.5	4903.1	5133.1	5239.2
		CV%	47.7%	45.6%	4.3%	8.3%	14.3%

39 bead. Consequently, to exceed the maximum sample value of 10 standard deviations above the mean of the negative sample value and to fall below the minimum sample value of 3 standard deviations below the mean of the positive sample value, the provisional cut-off value of HPV-DNA detection beads was set at 2000 for the HPV 39 bead and 1000 for the other HPV-type beads. The analytical sensitivity of the multiplex HPV primers was determined for all HPV types included in the assay, using 2-fold dilution series of plasmids containing control plasmid DNA. Duplicate samples were assayed thrice for each HPV type. DNA from all HPV types was detected in control plasmid DNA samples at 125, 250, 500, and 1000 copies/test (Table 3).

3.2. Mixed control plasmid sample study

To examine the performance of the assay in the detection of multiple HPV-type infections, HPV 16 plasmid DNA was mixed with other HPV-type plasmid DNAs at ratios ranging from 1:1 to 1:10,000. HPV 16 plasmid could not be detected in samples at a concentration of 250 copies/test, when other HPV-type plasmids were mixed at concentrations of more than 250×10^3 copies/test. In contrast, HPV 16 plasmid samples at concentrations of 250×10^4 copies/test could be detected without being influenced by the concentration level of the other HPV-type plasmids (Table 4).

3.3. Cross-reactivity study

To assess potential cross-reactivity with other bacteria and viruses, organism genomes were added to a β-globin control plasmid DNA sample. The microbial strains used were herpes simplex virus type 1, herpes simplex virus type 2, Epstein Barr virus, adenovirus type 2, cytomegalovirus, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Neisseria lactamica*, *Escherichia coli*, *Proteus vulgaris*, *Klebsiella pneumonia*, *Enterobacter cloacae* IFO3320, *Haemophilus ducreyi*, *Lactobacillus gasseri*, *Fusobacterium nucleatum*, *Chlamydia trachomatis*, *Candida albicans*, *Mycoplasma hominis*, *Mycoplasma hyorhinis*, *Ureaplasma urealyticum*, *Trichomonas vaginalis*, *Porphyromonas asaccharolytica*, *Gardnerella vaginalis*, *Treponema vincentii*, *Acinetobacter genomospecies*, *Mobiluncus curtisii*, and *Clostridium sticklandii*. Purified viral DNA was tested at 1×10^5 copies/test, and bacterial DNA was added at 1×10^5 cfu/ml. Only β-globin was detected in all samples; therefore, the presence of other organisms did not affect the detection of HPV DNA in this assay.

3.4. Genotypic specificity of the PCR products

To confirm that the PCR products were not non-specific, cloning and sequencing were performed. All 52 samples showed 99% or 100% identities on BLAST [accession numbers: type 18 (U89349), type 31 (U37410 and J04353), type 33 (U45897), type 35 (X74477), type 39 (U45905), type 45 (U45913), type 51 (CQ4877141), type 52 (HQ537743), type 56 (X74483), type 58 (U45929), type 59 (X77858), and type 68 (U45934)], confirming that the assay can be used for precise HPV genotyping (data not shown).

3.5. Clinical sample analysis

A total of 356 clinical samples were tested using the HPV genotyping kit. HPV-DNAs were detected in 162 (45.5%) samples, including 110 (67.9%) with single infection and 52 (32.1%) with multiple infections, of which 41 (78.8%) were double infections, 7 (13.5%) were triple infections, 3 (5.8%) were quadruple infections, and 1 (1.9%) was a quintuple infection. The type distribution of the 13 high-risk HPVs was as follows: 28.4% HPV 16, 11.7% HPV 18, 6.8%

Table 4
Mixed control plasmid sample study for detecting HPV 16 plasmid.

HPV type	Median fluorescence intensity							
	Plasmid mixing ratio (HPV 16: other 12 types of HPV)							
	1:1[a]	1:100	1:1000	1:10,000	10,000:1	10,000:100	10,000:1000	10,000:10,000
HPV 16	8610	1484	429	232	8908	8490	7315	1620
HPV 18	8360	6095	7826	10,689	9719	11,150	7669	1973
HPV 31	7196	4314	5381	7751	5655	6767	5283	1207
HPV 33	9533	4996	7425	10,709	8770	11,169	8915	1404
HPV 35	9027	2133	2749	3625	8702	5564	3275	497
HPV 39	3484	2455	3403	5267	3318	4720	3706	782
HPV 39	2968	1200	1613	1988	2620	2360	1933	271
HPV 39	6452	3655	5016	7255	5938	7080	5639	1053
HPV 45	6505	4122	5753	7845	6021	8018	5613	1236
HPV 51	7940	3914	7158	9441	6733	8866	6647	1528
HPV 52	10,675	4979	6120	72,526	10,990	10,754	8017	911
HPV 56	7663	4858	5607	8707	7246	8085	5343	1268
HPV 58	3609	2871	5977	8427	1953	5675	4883	1402
HPV 59	8000	4364	7276	9931	7737	8523	7367	2169
HPV 68	8444	5909	5834	8904	7096	8337	6146	1482
β-Globin	3233	948	1433	871	3488	2684	1339	111

[a] 250 copies of HPV 16: 250 copies of each 12 HPV type (total 3000 copies of HPVs).

HPV 31, 3.1% HPV 33, 3.7% HPV 35, 9.3% HPV 39, 1.9% HPV 45, 8.6% HPV 51, 37.0% HPV 52, 9.3% HPV 56, 16.7% HPV 58, 3.7% HPV 59, and 1.9% HPV 68.

3.6. Sample stability in liquid-based cytology media

The changes in MFI over time were measured in 3 types of liquid-based cytology media, with a cut-off value of 1000. DNA of HPV 16, HPV 18, and β-globin contained in HeLa or SiHa cells could be detected in Tacas and Thinprep samples stored at 4 °C or 30 °C for 4 weeks or less. In contrast, only HPV 18 was detected in Surepath samples stored at 4 °C or 30 °C for 4 weeks. HPV 16 could not be detected in Surepath samples stored at 4 °C for 4 weeks or 30 °C for 2 weeks. Furthermore, β-globin was not detected in Surepath samples stored at 4 °C or 30 °C for 1 week (Table 5).

4. Discussion

To assess the performance of the Mebgen HPV assay, recombinant plasmid DNA was used as a reference standard. In the investigation of the accuracy of the assay, MFI of beads was confirmed in triplicate measurements, and MFI of type-specific beads varied as much as 10- to 100-fold among the other beads. Therefore, the assay was considered to offer accurate type-specific detection. Using a cut-off value of 1000 for MFI (2000 for HPV 39), each DNA, expect HPV 68 DNA, was detected at 62.5 copies/test, and all types were detected at 125–1000 copies/test. Therefore, the detection limit of the assay was determined to be 125 copies/test, and 250 copies/test, provided enough fluorescence intensity to serve as the minimum detection sensitivity. In the examination of mixed HPV-type plasmids for multiple HPV-type infections, HPV 16 could not be detected in samples that included the minimum detection sensitivity concentration when DNA of other HPV types was added at concentrations greater than 250×10^3 copies/test. The reason for this is unknown, but competition for reagents can result in a loss of sensitivity and the detection of viral types present at lower copy numbers (Klug et al., 2008) and may lead even to false-negative results. The cloning and sequencing method, which is considered to obtain the most reliable results, confirmed the validity of the genotypic specificity of the amplified PCR products and confirmed the reliability of a positive result in this assay. The assay was tested also using plasmid control samples along with 27 other types of microbial DNA. There was no cross-reaction, demonstrating that the presence of other DNA mixed with HPV DNA in endocervical curettage specimens did not affect the performance of the assay.

The results of the sample stability test showed that the assay can be used successfully with the DNA of cultured cells extracted from Thinprep or Tacas samples stored at 4 °C or 30 °C for up to 4 weeks. However, Surepath samples should be tested within 1 week of collection because the findings suggested that nucleic acid degradation occurred during storage in the medium. Surepath medium is thought to be more challenging, primarily due to the formaldehyde crosslinking of proteins and nucleic acids (Moelans et al., 2011; Naryshkin and Austin, 2012). HeLa and SiHa cell lines contain 10–50 and 1–2 copies of HPV 18 and HPV 16 DNA/cell, respectively (Mincheva et al., 1987). According to the DNA concentration of the sample and the minimum detection sensitivity of the assay, the amount of detectable DNA decreased by 1/1000–1/10,000 over 4 weeks of storage. Further investigation is required to determine the appropriate long-term storage conditions for each liquid-based cytology media.

The 356 clinical samples used in this study were obtained from patients with persistently abnormal smears diagnosed at mass public health screenings for cervical cancer; therefore, a high positive rate (45.5%) of HPV was observed. However, many other researchers have reported previously the same genotype distribution found in this study. Consequently, this distribution is considered to be characteristic of the HPV-type distribution in Japan, with HPV 16 detected at a moderately low frequency and HPV 52 detected at a high frequency compared with the distributions in European countries (Miura et al., 2006). Additional statistical studies using new genotyping methods optimized for mass screening are needed to develop a more effective vaccine that matches the type distribution of the geographical region.

Currently, there are several commercially available HPV tests, but all have some limitations. HC2 cannot detect all genotypes of HPV, and as established previously, its detection limit is 5000 copies of HPV per assay (Lorincz, 1996). In addition, HC2 lacks internal standards to assess sample quality (Stoler et al., 2007). The Linear array™ HPV genotyping test/line-blot assay using a manual technique (Roche Diagnostics Gmbh, Mannheim, Germany) allows the detection of multiple infections and is very sensitive and well validated (Giuliani et al., 2006). However, it provides only semi-quantitative results as a visual read-out, which creates

Table 5
Change over time of median fluorescence intensity in each liquid-based cytology medium (cell concentration: 10^5 cell/ml).

Week	n	Stored at 4 °C			Stored at 30 °C			Stored at 4 °C			Stored at 30 °C		
		Thinprep	Surepath	Tacas	Thinprep	Surepath	Tacas	Thinprep	Surepath	Tacas	Thinprep	Surepath	Tacas
		HPV 16 from SiHa cells						β-Globin from SiHa cells					
0	1	8869	9270	8761	8761	8761	8761	3133	3097	3144	3144	3144	3144
	2	8934	8980	8748	8748	8748	8748	3316	3121	3139	3139	3139	3139
1	1	8810	2640	8392	8804	2584	8439	3387	95	3160	3410	198	2996
	2	9181	3558	9224	9144	3730	9229	3496	95	3527	3755	201	2860
2	1	9800	2927	9638	8883	2745	9991	3777	105	3805	3452	86	2737
	2	9538	3239	9153	9989	893	9966	3723	102	3668	3919	163	2891
3	1	9579	2591	8200	9872	61	7384	3894	177	3298	3856	117	1785
	2	10,333	2603	7263	10,089	68	6796	4039	132	2706	3892	116	1519
4	1	7435	57	6338	6896	30	8078	2432	81	2322	2699	79	1539
	2	6698	1744	7743	7504	916	7809	2439	82	2936	2810	114	1489
		HPV 18 from HeLa cells						β-Globin from HeLa cells					
0	1	11,772	11,079	10,796	10,796	10,796	10,796	2697	2361	2546	2546	2546	2546
	2	11,985	11,337	12,505	12,505	12,505	12,505	2670	2416	2703	2703	2703	2703
1	1	11,618	11,254	12,490	12,335	11,042	12,425	2938	171	3075	3214	166	2892
	2	12,546	11,507	13,265	12,566	10,651	12,925	3329	204	3089	1682	129	2858
2	1	12,725	11,683	12,893	13,331	11,427	14,420	3438	150	3252	3367	108	3007
	2	13,154	11,876	13,254	13,605	10,085	14,383	3481	158	3547	3278	134	2901
3	1	13,278	11,869	14,647	13,699	10,546	12,436	3555	105	3360	3188	85	2394
	2	13,758	11,779	13,718	13,100	9788	14,480	3702	119	2992	2950	77	2501
4	1	10,096	8644	9849	10,841	8967	11,457	2694	183	2321	2674	86	2157
	2	9595	9083	9428	10,585	7821	10,667	2436	95	2135	2387	62	1792

problems with reproducibility and increase the risk of data entry errors (Schmitt et al., 2006). The polymerase chain reaction method using MY09/MY11 and/or GP5+/GP6+ primers has been used widely in the past decade (Castle et al., 2002; Fuessel Haws et al., 2004). These consensus primers are able to detect a broad spectrum of HPV types, but because of biased amplification, they are not suitable for genotyping in samples containing multiple HPV types (Mori et al., 2011). The Amplicor™ HPV test (Roche Diagnostics Gmbh) is an enzyme-linked immunosorbent assay that involves a manual technique. This test is useful for small laboratories, but it is difficult to apply in mass screening. Recently, a number of new genotyping kits for dedicated measuring instruments have been commercialized [e.g., the Cobas™ HPV test (Roche Diagnostics Gmbh), the Cervista™ HPV HR test (Hologic), and the Abbott Realtime high risk HPV test (Abbott Laboratories, IL, USA), all of which use real-time PCR assay, and the Clinichip™ HPV (Sekisui medical, Tokyo, Japan), which uses an automated DNA chip system)]. Further, comparative testing is necessary to assess the relative advantages of these systems.

The Mebgen HPV assay uses the Luminex system to detect specific PCR products in a 96-well format. As a result of the advantages of the high-throughput screening method, this system has gained already a solid reputation in various fields of genotyping (e.g., inflammatory cytokines, bacterial pathogens, and single nucleotide polymorphisms) (Armstrong et al., 2000; Dunbar et al., 2003; Skogstrand et al., 2005; Ye et al., 2001). In addition to the use of the reliable Luminex system, this HPV genotyping kit uses dUTP in place of dTTP in the dNTP mix, and UDG enzyme is added to the PCR master mix. Because UDG does not react with dUTP and is inactivated also by heat denaturation before the actual PCR, carry-over contamination of PCRs can be controlled effectively if the contaminants contain uracil in place of thymine (Longo et al., 1990). For HPV genotyping, an appropriate assay should be selected on the basis of the specific purpose and aims of each laboratory. The new Mebgen HPV genotyping kit offers another valuable option for use in the future.

5. Conclusion

The present findings suggest that the Mebgen HPV kit can be potentially used for clinical applications and large-scale epidemiological and vaccination studies. Further studies with larger sample sizes and a comparative study with another assay are warranted.

Conflict of interest

This study was supported by Medical & Biological Laboratories (MBL), Tokyo, Japan. The Mebgen HPV kit was provided by MBL. Study-related work was performed by G&G Science. HH and HK are employees of MBL. KS and YA are employees of G&G Science. All other authors have declared that no conflicts of interest exist. The source of funding had no influence on the analyses or interpretation of the results presented in the paper.

Acknowledgments

The authors sincerely thank Etsuko Futaya (MBL Corporation), Masahiko Okada (Kanazawa University), and the staff of the Division of Pathology, Kanazawa University Hospital and Ohasi Medical Center for their technical support.

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